U.S. DEPARTMENT OF COMMERCE
National Technical Information Service

PB-256 930

MISSOURI RIVER BASIN STEROL ASSAY PROJECT REPORT. COPROSTANOL, A POSITIVE MARKER OF DOMESTIC AND RUN-OFF POLLUTION

STEROL ASSAY OF WASTEWATER PLANT EFFLUENTS AND SURFACE WATERS OF THE LOWER MAIN STEM MISSOURI

FEDERAL WATER QUALITY ADMINISTRATION

25 MAY 1970

CO2209 246095 PB 256 930

MISSOURI RIVER BASIN STEROL ASSAY PROJECT REPORT

COPROSTANOL, A POSITIVE MARKER OF DOMESTIC AND RUN-OFF POLLUTION

Sterol Assay of Wastewater Plant Effluents and Surface Waters of the Lower Main Stem Missouri

Henry H. Tabak and Robert L. Bunch

U. S. DEPARTMENT OF THE INTERIOR
Federal Water Quality Administration
Advanced Waste Treatment Research Laboratory
Cincinnati, Ohio
May 25, 1970

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U. S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

BIBLIOGRAPHIC DATA SHEET	1. Report No.	2.	3. Recipient's Accession No. PB-256 930
4. Title and Subtitle: Missouri River Basin Sterol Assay Project Report. Coprostanol, A Positive Marker of Domestic and Run-Off Pollution. Sterol Assay of Wastewater Plant Effluents and Surface Waters of the Lower Main Stem Missouri.			5. Report Date: May 25, 1970 6.
7. Author(s): Henry H. Tabak, and Robert L. Bunch.			8. Performing Organization Rept. No.
9. Performing Organization Name and Address: Federal Water Quality Administration Cincinnati, Ohio Advanced Waste Treatment Research Laboratory			10. Project/Task/Work Unit No. 11. Contract/Grant No.
12. Sponsoring Organization Name and Address			13. Type of Report & Period Covered 14.

15. Supplementary Notes

16. Abstracts: This study was undertaken to estimate the extent of human and warm-blooded animal fecal pollution and to pinpoint the main sources of this pollution in the Missouri River. The survey covered 28 sampling stations in the Sioux City, Omaha, St. Joseph and Kansas City area. The analytical method used for the estimation of the coprostanol in the river waters and in the wastewaters was based on the methodology which was previously developed by Murtaugh and Bunch. There was one major change in that a new packing was used for the gas-liquid chromatography column which permitted the free sterols to be run. This eliminated the need for making the trimethylsilyl ether derivatives. The procedure consisted of hexane extraction, mild alkali-alcohol hydrolysis of esters and conjugates to free sterols, cleanup by thin-layer chromatography (TLC) and quantitative measurement by gas-liquid chromatography (GLC). Simultaneously with the assay for the fecal sterol the number of fecal coliforms were determined utilizing the membrane filter technology.

17. Key Words and Document Analysis. 17a. Descriptors

*Sewage
Runoff
Sterols
Effluents
Missouri River
Sampling
Concentration(Composition)
Membranes
Filters
Gas chromatography
Extraction
Thin layer chromatography
Marking
Water quality

17b. Identifiers/Open-Ended Terms

*Cholestanols
*Coprostanols
*Water quality data

17c. COSATI Field/Group 13B

18. Availability Statement: National Technical Information Service Springfield, VA 22161	19. Security Class (This Report) UNCLASSIFIED	21. No. of Pages 18
	20. Security Class (This Page) UNCLASSIFIED	22. Price PC$3.50/ MF$2.25

INSTRUCTIONS FOR COMPLETING FORM NTIS-35 (Bibliographic Data Sheet based on COSATI Guidelines to Format Standards for Scientific and Technical Reports Prepared by or for the Federal Government, PB-180 600).

1. **Report Number.** Each individually bound report shall carry a unique alphanumeric designation selected by the performing organization or provided by the sponsoring organization. Use uppercase letters and Arabic numerals only. Examples FASEB-NS-73-87 and FAA-RD-73-09.

2. Leave blank.

3. **Recipient's Accession Number.** Reserved for use by each report recipient.

4. **Title and Subtitle.** Title should indicate clearly and briefly the subject coverage of the report, subordinate subtitle to the main title. When a report is prepared in more than one volume, repeat the primary title, add volume number and include subtitle for the specific volume.

5. **Report Date.** Each report shall carry a date indicating at least month and year. Indicate the basis on which it was selected (e.g., date of issue, date of approval, date of preparation, date published).

6. **Performing Organization Code.** Leave blank.

7. **Author(s).** Give name(s) in conventional order (e.g., John R. Doe, or J.Robert Doe). List author's affiliation if it differs from the performing organization.

8. **Performing Organization Report Number.** Insert if performing organization wishes to assign this number.

9. **Performing Organization Name and Mailing Address.** Give name, street, city, state, and zip code. List no more than two levels of an organizational hierarchy. Display the name of the organization exactly as it should appear in Government indexes such as Government Reports Index (GRI).

10. **Project/Task/Work Unit Number.** Use the project, task and work unit numbers under which the report was prepared.

11. **Contract/Grant Number.** Insert contract or grant number under which report was prepared.

12. **Sponsoring Agency Name and Mailing Address.** Include zip code. Cite main sponsors.

13. **Type of Report and Period Covered.** State interim, final, etc., and, if applicable, inclusive dates.

14. **Sponsoring Agency Code.** Leave blank.

15. **Supplementary Notes.** Enter information not included elsewhere but useful, such as: Prepared in cooperation with . . . Translation of . . . Presented at conference of . . . To be published in . . . Supersedes . . . Supplements . . . Cite availability of related parts, volumes, phases, etc. with report number.

16. **Abstract.** Include a brief (200 words or less) factual summary of the most significant information contained in the report. If the report contains a significant bibliography or literature survey, mention it here.

17. **Key Words and Document Analysis. (a). Descriptors.** Select from the Thesaurus of Engineering and Scientific Terms the proper authorized terms that identify the major concept of the research and are sufficiently specific and precise to be used as index entries for cataloging.
(b). Identifiers and Open-Ended Terms. Use identifiers for project names, code names, equipment designators, etc. Use open-ended terms written in descriptor form for those subjects for which no descriptor exists.
(c). COSATI Field/Group. Field and Group assignments are to be taken from the 1964 COSATI Subject Category List. Since the majority of documents are multidisciplinary in nature, the primary Field/Group assignment(s) will be the specific discipline, area of human endeavor, or type of physical object. The application(s) will be cross-referenced with secondary Field/Group assignments that will follow the primary posting(s).

18. **Distribution Statement.** Denote public releasability, for example "Release unlimited", or limitation for reasons other than security. Cite any availability to the public, other than NTIS, with address, order number and price, if known.

19 & 20. **Security Classification.** Do not submit classified reports to the National Technical Information Service.

21. **Number of Pages.** Insert the total number of pages, including introductory pages, but excluding distribution list, if any.

22. **NTIS Price.** Leave blank.

COPROSTANOL, A POSITIVE MARKER OF DOMESTIC AND RUN-OFF POLLUTION

Sterol Assay of Wastewater Plant Effluents and Surface Waters of the Lower Main Stem Missouri

Henry H. Tabak and Robert L. Bunch

The specific proof of pollution of natural waters from various sources requires isolation and identification of the specific organic pollutants in these waters. Although contamination of waters by domestic sewage is commonly concluded on the basis of standard coliform organism counts, the use of a specific fecal organic compound that can be correlated quantitatively with this major source of pollution is more desirable. Steroids offer such a class of compounds, since certain steroids are characteristic of wastes from human beings and higher animals.

Coprostanol (coprostan-3-β-ol, 5 β-cholestan-3 β-ol) is one of the principal sterols in feces of human beings and higher animals, and feces are said to be the only source of this compound. The finding of coprostanol in water would therefore indicate excreta from either domestic wastes or run-off from pastures or barnyards. The merits of using this major human fecal sterol as a positive marker of domestic pollution has been recently emphasized (Murtaugh and Bunch, 1967; and Smith and Gouron, 1969). It was shown by the first authors that these compounds are removed by adequate secondary sewage treatment.

This study was undertaken to estimate the extent of human and warm-blooded animal fecal pollution and to pinpoint the main sources of this pollution in the Missouri River. The survey covered 28 sampling stations in the Sioux City, Omaha, St. Joseph and Kansas City area. The sampling stations and their river mileage (RM) are described in appendix I. Each location was sampled four different times at approximately two-week intervals. The samples were iced and flown to this laboratory. They were about one day old when analyzed.

The analytical method used for the estimation of the coprostanol in the river waters and in the wastewaters was based on the methodology which was previously developed in this laboratory (Murtaugh and Bunch, 1967), and proven effective for the recovery of sterols from wastewater as well as from a stream in the Cincinnati area. There was one major change in that a new packing was used for the gas-liquid chromatography column which permitted the free sterols to be run. This eliminated the need for making the trimethylsilyl ether derivatives. The procedure consisted of hexane extraction, mild alkali-alcohol hydrolysis of esters and conjugates to free sterols, cleanup by thin-layer chromatography (TLC) and quantitative measurement by gas-liquid chromatography (GLC). The procedure used is fully described in appendix II.

Simultaneously with the assay for the fecal sterol the number of fecal coliforms were determined utilizing the membrane filter technology.

Results and Discussion

The examination of sewage effluents and surface waters in the Sioux City, Omaha, St. Joseph and Kansas City areas of the lower main stem

Missouri revealed the presence of coprostanol. The chromatographic analyses of all samples from the sampling points show significant variations in the amount of the specific indicator of domestic pollution, coprostanol, as the degree of sewage pollution of the watercourse varied.

The sewage treatment plant (STP) effluents along the Missouri River within the above four sampling areas were shown to have significantly higher concentration of the fecal sterol than the surface water samples taken at points just before the plant sewage effluent discharge area or further down the river away from the treatment plants. The decrease in concentration of coprostanol was demonstrated adequately by analysis of water samples taken further away along the riverfrom the location of treatment plant outfalls.

Table 1 summarizes the analytical data on the concentration of coprostanol in effluent and surface water samples from the 28 sampling points. The treated sewage effluent samples from the Sioux City treatment plant (SC-49) contained a concentration of coprostanol in the range of 636 to 794 µg per liter. The STP effluents of the Omaha area at sampling points CB-40 B and OM-40 A gave values in the range of 743 to 864 and 250 to 362 µg per liter respectively. The range values of coprostanol concentration in samples of sewage effluent from sampling points SJ-15, SJ-18, A-25.5 and L-24.5 of the St. Joseph area were, respectively, 391 to 484, 465 to 573, 365 to 498 and 424 to 535 µg per liter. Kansas City area samples of STP effluent from M-103, M-104, and M-19 sampling points contained concentrations of fecal sterol in the range from 496 to 587, 259 to 319, and 328 to 419 µg per liter, respectively. The surface river

water samples contained substantially lower concentrations of coprostanol, particularly at points further away from the treatment plant outfalls.

These conclusions are further substantiated by the microbiological data compiled for the same samples which were subjected to sterol analysis. A resume on the enumeration of fecal coliforms in the treated sewage effluents and surface river samples from the Missouri River is given in Table 2. The treated sewage effluents demonstrate the number of fecal coliforms in the millions per 100 ml of sample, while surface river samples further away from the plant sewage outfall show a progressively lower density of fecal coliforms. Since the same trend was determined for the concentration of coprostanol in the effluent and surface river samples, there seems to be a definite and significant correlation between the concentration of the fecal sterol and the degree of fecal pollution. This is graphically shown in Figure 1 which is a profile of the average density of fecal coliforms and average concentration of coprostanol for the 28 sampling stations.

On an average a hog, a cow or a human being would be expected to excrete 800 to 1,000 mg of coprostanol per day. If this is diluted with 100 gallons of wastewater, the expected concentration would be 210 to 250 µg per liter in raw wastewater. The results as compiled in Table 1 show that all treatment plant effluents exceeded this amount. The river samples are very high considering the dilution the wastewater would receive upon entering the river. The Missouri River samples were more than 10-fold greater in coprostanol than below an outfall of a poorly run secondary treatment plant in the Cincinnati area (Murtaugh and Bunch, 1967).

Summary

Sewage treatment plant effluents and surface river water samples from 28 sampling stations on the lower main stem Missouri were analyzed for the presence of the fecal sterol, coprostanol.

Data compiled on the amounts of coprostanol in the treatment plant effluents and surface waters indicate conclusively that the Missouri River is polluted with fecal matter.

Significant variations in the amount of coprostanol in the effluents and surface waters at sampling points further away from sewage effluent outfalls were clearly demonstrated and these show a correlation to the density of fecal coliforms at the same locations.

The analytical data definitely demonstrate the potential for using the analysis for sterol content to measure fecal pollution and the merit for using coprostanol as a positive marker of fecal pollution. The specific nature of coprostanol and its occurrence in surface waters polluted by domestic and run-off sewage leave little doubt that the presence of this fecal sterol connotes fecal pollution and that it ought to be used as an index of pollution in addition to the standard method for enumeration of fecal coliforms.

Acknowledgements

The authors wish to acknowledge the excellent assistance of the personnel of the Missouri Basin Region of FWQA for the laborious task of furnishing all the samples for this study. The authors express their

sincere appreciation to Mr. Robert N. Bloomhuff for his enthusiasm and extremely capable assistance throughout the development of this study and for the photographic work on thin-layer chromatographs. The authors also wish to express their thanks to Mr. Cecil W. Chambers and Mr. Albert D. Venosa for supplying microbiological data of this study.

References

Murtaugh, J. and Bunch, R. L. 1967. Sterols as a measure of fecal pollution. J. Water Poll. Cont. Fed., 39:3, 404-409.

Smith, L. L. and Gouron, R. E. 1969. Sterol metabolism - VI. Detection of 5 β-cholestan-3 B-ol in polluted waters. Water Research, Pergamon Press, Vol. 3, pp. 141-148. Printed in Great Britain.

TABLE 1. Quantitative Analysis of Coprostanol in Waste Treatment Plant Effluent and Surface Water Samples Taken from Sampling Points of the Lower Main Stem Missouri

Sampling Area	Sampling Point	River Mileage	Concentration of Coprostanol in µg/liter				Mean
			Date of Sampling				
			1-20-70	3-4-70	3-17-70	3-24-70	
Sioux City Area	M-52	736.0	4	6	10	3	6
	SC-49	729.0	636	723	684	793	709
	M-48	717.4	98	105	93	109	101
	B-43	631.1	64	66	60	57	62
	M-42	626.2	21	16	23	20	20
			1-27-70	2-23-70	3-10-70	3-31-70	
Omaha Area	M-42	626.2	9	20	26	28	21
	CB-40 B	614.0	743	864	766	815	797
	OM-40-A	611.5	250	300	362	335	312
	M-38	601.7	73	71	76	67	72
	M-205	596.6	177	200	295	166	210
	P-37	594.8	17	21	13	15	16
	M-34	559.7	60	73	76	72	70
	M-28	452.3	31	34	31	37	33
			2-4-70	4-7-70	4-21-70	5-5-70	
St. Joseph Area	M-28	452.3	37	31	35	28	33
	SJ-15	446.4	391	418	484	452	436
	SJ-18	445.6	573	465	503	493	508
	M-27	440.3	56	49	48	43	49
	A-25.5	421.0	365	451	498	389	424
	L 24.5	395.6	424	519	486	535	491
	M-23	370.5	63	46	54	60	56

1. (Continued)

Sampling Area	Sampling Point	River Mileage	Concentration of Coprostanol in µg/liter — Date of Sampling				Mean
			2-11-70	4-14-70	4-28-70	5-13-70	
Kansas City Area	M-23	370.5	80	68	98	--	82
	K-22	367.4	78	70	83	--	77
	M-103	367.20	522	496	587	--	535
	M-104	367.19	259	319	298	--	290
	M-107 B	358.0	92	110	92	--	95
	M-19	358.0	328	419	396	--	381
	M-18	345.4	86	75	98	--	87
	M-15	293.0	66	79	58	--	70

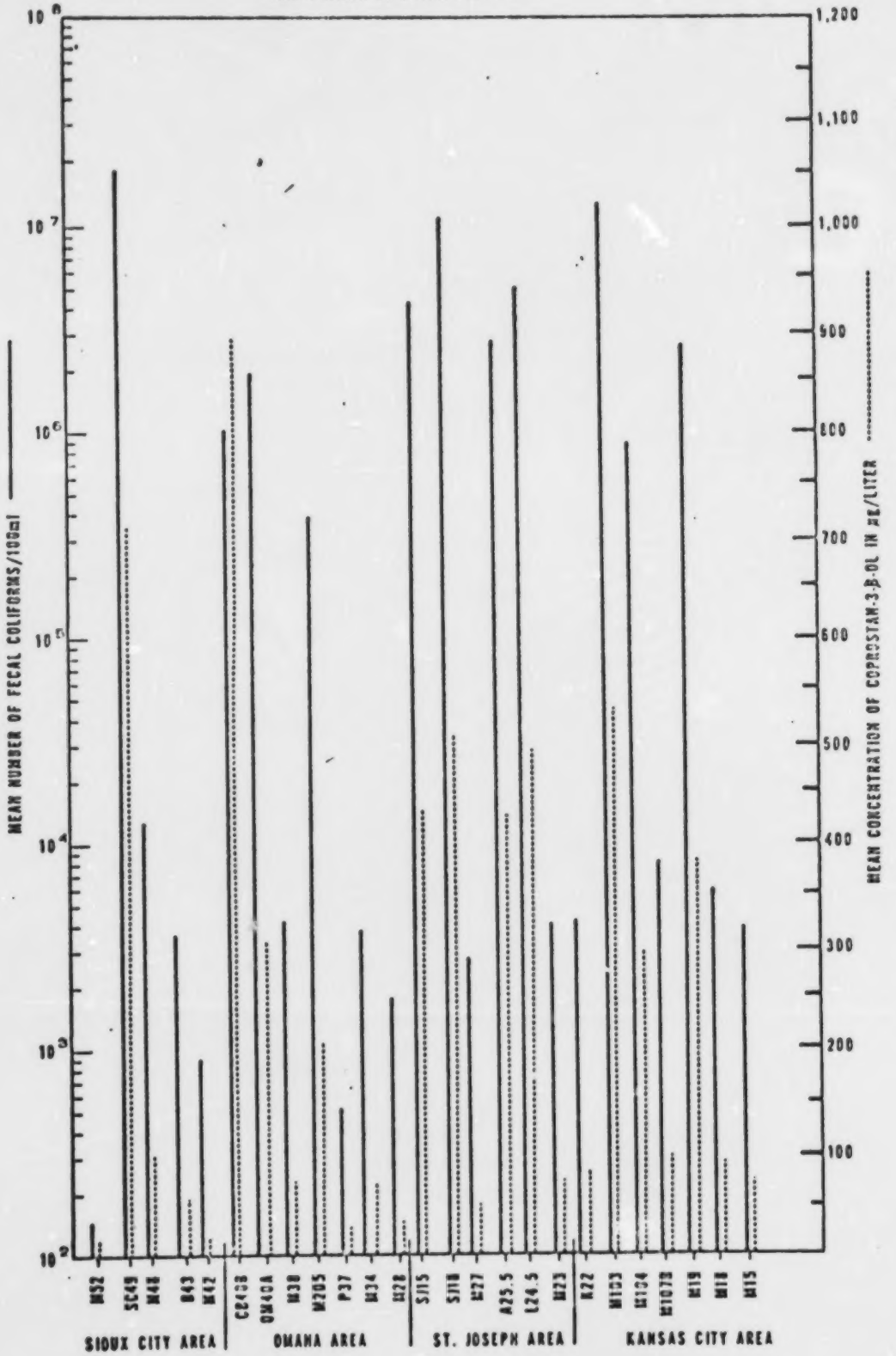
TABLE 2. Enumeration of Fecal Coliforms in Waste Treatment Plant Effluent and Surface Water Samples Taken from Sampling Points at the Lower Main Stem Missouri

Sampling Area	Sampling Point	River Mileage	Number of Fecal Coliforms per 100 ml of Sample — Date of Sampling				Mean
			1-20-70	3-4-70	3-17-70	3-24-70	
Sioux City Area	M-52	736.0	---	360	8	44	140
	SC-49	729.0	---	26×10^6	20×10^6	12×10^6	19×10^6
	M-48	717.4	---	1.4×10^4	0.8×10^4	1.6×10^4	1.3×10^4
	B-43	635.1	---	1.0×10^4	380	705	3,500
	M-42	626.2	---	---	930	600	770
			1-27-70	2-23-70	3-10-70	3-31-70	
Omaha Area	M-42	626.2	---	1,200	800	1,000	1,000
	CB-40 B	614.0	---	2.9×10^6	1.7×10^6	4.0×10^6	2.9×10^6
	OM-40 A	611.5	---	1.0×10^6	0.8×10^6	4.0×10^6	1.9×10^6
	M-38	601.7	---	3,000	3,000	6,700	4,200
	M-205	596.6	---	3.1×10^5	4.1×10^5	4.1×10^5	3.8×10^5
	P-37	594.8	---	340	540	730	540
	M-34	559.7	---	1,800	3,700	5,800	3,800
	M-28	452.3	---	2,400	1,800	1,900	2,100
			2-4-70	4-7-70	4-21-70	5-5-70	
St. Joseph Area	M-28	452.3	1,600	1,400	2,600	560	1,800
	SJ-15	446.4	1.6×10^6	6.8×10^6	0.7×10^6	7.8×10^6	4.2×10^6
	SJ-18	445.6	1.4×10^6	4.2×10^6	22×10^6	17×10^6	11×10^6
	M-27	440.3	5,200	1,800	3,700	450	2,800
	A-25.5	421.0	0.97×10^6	5.0×10^6	0.27×10^6	4.9×10^6	2.8×10^6
	L-24.5	375.6	4.4×10^6	5.8×10^6	1.9×10^6	11×10^6	5.7×10^6
	M-23	370.5	6,200	2,400	3,000	1,000	3,150

2. (Continued)

Sampling Area	Sampling Point	River Mileage	Number of Fecal Coliforms per 100 ml of Sample				Mean
			Date of Sampling				
			2-11-70	4-14-70	4-28-70	5-13-70	
Kansas City Area	M-23	370.5	880	2,100	700	18,500	5,500
	K-22	367.4	6,200	1,300	7,400	1,800	4,200
	M-103	367.20	20×10^6	13×10^6	5.9×10^6	13×10^6	13×10^6
	M-104	367.19	1.3×10^6	0.3×10^6	0.4×10^6	1.6×10^6	0.9×10^6
	M-107 B	358.0	80	500	400	32,000	8,300
	M-19	358.0	1.9×10^6	2.6×10^6	2.2×10^6	4.1×10^6	2.7×10^6
	M-18	345.4	1,600	1,600	2,100	19,000	6,100
	M-15	293.0	770	1,800	1,600	11,800	4,000

RELATIONSHIP BETWEEN CONCENTRATION OF COPROSTAN-3-β-OL
AND NUMBER OF FECAL COLIFORMS



APPENDIX I

SAMPLING LOCATIONS - LOWER MAIN STEM MISSOURI

Sioux City Area

1. M-52 Missouri River at RM-736 above the mouth of the Big Sioux River
2. SC-49 Sioux City STP Effluent
3. M-48 Sioux City mixing zone station - Missouri River at RM 717
4. M-42 Missouri River at RM 626.2, Omaha Waterworks Intake
5. B-43 Boyer River at I-29 Highway Bridge above Omaha

Omaha Area

1. M-42 Missouri River at RM-626.2, Omaha Waterworks Intake
2. OM-40A Omaha-Missouri River STP Effluent
3. CB-40B Council Bluffs STP Effluent
4. M-205 Papillion Creek at CRB 1 mile below US-73
5. M-38 Missouri River at Bellevue RM-601.6, Omaha area mixing station
6. M-34 Missouri River at Nebraska City RM-559.7
7. M-28 Missouri River at St. Joseph Waterworks Intake RM-452.3
8. P-37 Platte River at US-73 Bridge Junction at RM-594.9L

St. Joseph Area

1. M-28 Missouri River at St. Joseph Waterworks Intake RM-452.3
2. SJ-15 St. Joseph Municipal STP effluent
3. SJ-18 South St. Joseph Industrial STP effluent
4. M-27 Missouri River at St. Joseph area mixing station RM-440.3

5. A-25.5 Atchison STP effluent
6. L-24.5 Leavenworth STP effluent
7. M-23 Missouri River at Kansas City Waterworks Intake RM-370.5

Kansas City Area

1. M-23 Missouri River at Kansas City Waterworks Intake RM-370.5
2. K-22 Kansas River at Central Avenue Bridge in Kansas City, Kansas
3. M-103 Kansas City, Kansas STP Effluent Westside
4. M-104 Kansas City, Missouri STP Effluent
5. M-19 Kansas City, Missouri-Blue River STP Effluent
6. M-107B Big Blue River at Mouth RM-358.0
7. M-18 Missouri River at Missouri City Power Plant Mixing Zone
8. M-15 Missouri River at Waverly RM-293.0

APPENDIX II

METHODOLOGY

Analysis of Cholesterol and Coprostanol in Rivers and Wastewaters

I. Hexane extraction of sterols and sterol conjugates.

1. To 1 liter samples add 20 ml of conc. HCl and 20 ml of 20% NaCl aqueous solution. Mix thoroughly.
2. Extract three times with 1/5 volumes of prepurified hexane. Extraction is performed in 2 liter capacity separatory funnels on a reciprocal shaker.
3. The pooled hexane fractions are then passed through a column of sodium sulfate to eliminate the water content.
4. The hexane is evaporated in a flash evaporator under vacuum to near dryness.

II. Mild alkali-ethanol hydrolysis of hexane extracted sterols.

1. The near dry residue is transferred to small digestion flasks.
2. Add 3 ml of 25% KOH aqueous solution and mix the contents thoroughly.
3. Add 7 ml of 95% ethyl alcohol and mix the contents thoroughly.
4. Add a few boiling chips to each of the digestion flasks and place them on a hot plate, connect them to the condensers and hydrolyze the contents for 2 hours.
5. After hydrolysis, cool the flasks immediately in an ice bath.

III. Hexane extraction of hydrolysates.

1. To the chilled contents in digestion flasks add 10 ml of distilled water.
2. Transfer the contents to 125 ml capacity separatory funnels.
3. Rinse the digestion flasks thoroughly with 40 ml of prepurified hexane and pour the solvent into the separatory funnels.
4. Extract on reciprocal shaker.
5. Repeat extraction with second 40 ml volume of hexane.
6. The pooled two hexane fractions are rinsed with 10 ml of 2% aqueous NaCl solution, shaken thoroughly, and allowed to stand for 10 minutes.
7. The NaCl layer is drained off and the hexane layer is transferred to evaporation flasks and evaporated in a flash evaporator to almost dryness.

IV. Transfer of hydrolyzed extract to small storage tubes.

1. The near dry residue in evaporation flasks is rinsed 3 times with 2 ml volumes of absolute ethanol, each rinsing facilitated by 10-minute shaking on a reciprocal or rotary shaker.
2. The rinsings are placed into small tubes and the contents are evaporated to dryness under forced prepurified air on a specially designed steam bath.
3. Follow the rinsing of the evaporation flasks with two additional acetone rinsings using 2 ml of acetone for each rinse.
4. Acetone rinsings are evaporated in the tubes, using above method.

V. Preliminary thin layer chromatography of the sterol extracts.

1. 20 by 20 cm glass plates are coated with 0.25 mm layer of Silica Gel G (E. Merck, Darmstadt) and activate for one hour at 110 C.
2. The prepared plates are stored in a vacuum desiccator until used.
3. Standard solutions of the sterols in acetone are prepared as 0.1% solutions, and the reference standards are spotted by means of Hamilton syringes on the Silica Gel G plates, using 5 ug/5 ul twice per each spot.
4. To the hydrolyzed samples of extracted sterols in small tubes are added 0.5 ml volumes of warm acetone. The contents are dissolved in acetone and 10 ul volumes of each of the extract samples are spotted on the same plate next to the reference standards.
5. The drying of the spots is facilitated by the use of infrared lamp.
6. The chromatoplates are developed with a mixture of chloroform and ether in the ratio of 9:1. They are then air dried for 15 minutes and heat dried (110 C) for additional 5 minutes.
7. The chromatoplates are sprayed with a 10% solution of phosphomolybdic acid in 95% ethanol.
8. After heating at 100 C for 15 minutes, the sterols appear as dark spots (violet-blue-indigo) on a yellow background.
9. The Rf values of the standard references are compared with those of the sterol extracts.

VI. Preparative thin layer chromatography for GC chromatography analysis.

1. Reference standards of sterols are spotted on the plate using the same concentrations per spot as above.
2. 0.1 ml volumes of the hydrolyzed samples of extracted sterols in acetone are streaked on the same plates with the standards on the right half portions of each plate.
3. After drying, the chromatoplates are developed with a mixture of $CHCl_3$-ether in the ratio of 9:1, air dried and heat dried.

4. The sterols are located by covering that portion of the plate containing the unknown samples with aluminum foil and spraying the reference standards with 10% solution of phosphomolybdic acid in 95% ethanol.
5. The standard reference spots are visualized after heating at 100 C for 15 minutes.
6. Areas for unknown samples corresponding to locations of reference standards are removed with a micro spatula, placed in prewashed medicine droppers plugged with prewashed glass wool and eluted into micro thistle tubes with 5 ml of acetone.
7. The eluted sterols are concentrated in the thistle tubes to the top level of the thistle on a steam bath with the aid of a gentle stream of clean, dry air.

VII. Gas-liquid chromatography of the silica Gel G eluted sterols.

1. Quantitative determinations are made with an Aerograph gas chromatograph and using a flame ionization detector.
2. A stainless steel column 1/8 in x 6 ft is packed with 60-80 mesh chromasorb Q coated with fluoroalkyl silicone polymer QF-1 (3% by weight) and column temperature is maintained at 235 C.
3. Helium is used as carrier gas, helium pressure adjusted to 25 psi, and the carrier gas flow rate maintained at 48 ml per minute.
4. Hydrogen flow rate to the flame ionization detector is kept at 25 ml/min. and the source of hydrogen is a hydrogen generator.
5. The air flow to the ionization detector is maintained at 200 ml/min.
6. The temperatures of the injector and detector are kept at 240 C.
7. 5 ug/5 ul concentrations of reference standards are injected and standard reference peaks determined for the concentrations of each of the standard sterols injected.
8. The retention time of each of the respective sterol standards is determined for the above set of column and detector conditions used.
9. Quantitative determinations of the unknown sterol extracts are based on peak height compared to a standard curve prepared from known reference standards. Qualitative determinations of unknowns are based on the retention time as compared with that of standards.
10. Internal standards are employed in the analysis of each of the separated (TLC) unknown sterol fractions to definitely categorize the unknown sample.